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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A61K 39/395, C07K 13/00 C12N 15/13	A1	(11) International Publication Number: WO 91/19515 (43) International Publication Date: 26 December 1991 (26.12.91)
(21) International Application Number: PCT/US91/04395 (22) International Filing Date: 21 June 1991 (21.06.91) (30) Priority data: 541,527 21 June 1990 (21.06.90) US (71) Applicant: THE BOARD OF TRUSTEES OF THE LE- LAND STANFORD JUNIOR UNIVERSITY [US/ US]; Stanford University, Stanford, CA 94305 (US). (72) Inventors: SHOPES, Robert ; 7575 Eads, #E, La Jolla, CA 92037 (US). OI, Vernon, T. ; 1259 Marilyn Court, Moun- tain View, CA 94040 (US). STRYER, Lubert ; 843 Sono- ma Terrace, Stanford, CA 94305 (US).		(74) Agents: ROWLAND, Bertram, I. et al.; Cooley Godward Castro Huddleson & Tatum, Five Palo Alto Square, 4th Floor, Palo Alto, CA 94306 (US). (81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European pa- tent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (Euro- pean patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i>
(54) Title: OLIGOMERIC IMMUNOGLOBULIN CONSTANT DOMAIN MUTANT WITH ENHANCED COMPLE- MENT-MEDIATED CYTOLYTIC ACTIVITY (57) Abstract Oligomeric immunoglobulins joined at the constant region at a specific site provide for enhanced cytotoxicity in conjunction with complement. The subject compositions are shown to be effective in complete cytotoxicity at substantially lower concentrations than required with the monomeric immunoglobulin. The immunoglobulins are mutagenized by providing a cysteine in the third heavy domain, whereupon expressing a dimeric product is obtained.		

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OLIGOMERIC IMMUNOGLOBULIN CONSTANT DOMAIN MUTANT
WITH ENHANCED COMPLEMENT - MEDIATED CYTOLYTIC ACTIVITY

5

INTRODUCTION

Technical Field

10 The field of this invention is the use of
immunoglobulins in binding to cells.

Background

15 A number of modalities, both therapeutic and in
culture, utilizing monoclonal antibodies involve the
purging of undesired cells. Immunoglobulin induced
cytotoxicity can be attributed to a number of antibody
dependent effector functions. One of these is the well-
20 documented classical complement cascade initiated by an
activated immune complex which leads to lysis of the
antigen bearing cell. The molecular mechanism of the
activation of complement has long been a source of
speculation. Whether or not aggregation of antibody is
sufficient is still an open question but it is generally
25 thought that aggregation is necessary for initiation of
the cascade. Aggregated antibody in the absence of
antigen (e.g. heat treatment) can effectively activate
the complement cascade. However, this is not useful in
therapy. What would be useful is an immunoglobulin
30 which displays little or no activation of complement in
the absence of antigen binding and yields enhanced lysis
of the target cell population. It would therefore be of
interest to provide immunoglobulins or other proteins
having specific binding capability as well as
35 complement effector functions with enhanced

Relevant Literature

- Waldmann (Ed.) Monoclonal Antibody Therapy; Progress in Allergy Vol. 45 (Karger, New York, 1988);
- 5 Glassy and Dillman, Molecular Biotherapy 1,7 (1988), describe the use of monoclonal antibodies for the purging of undesired cells. There has been much speculation about the molecular mechanism of the activation of complement. Law and Reid, Complement,
- 10 (IRL Press, Washington D.C., 1988) Metzger, Contemp. Topics Mol. Imm. 7,191 (1978); Davies and Metzger, Ann. Rev. Immunol. 1,87 (1983); Bennett and Huber, CRC Crit. Rev. Biochem. 15,291 (1984); Burton, Mol. Immunol. 22,161 (1985); and Metzger, Harvey Lectures 80,49
- 15 (1986). The use of disulphide bonds for structural alteration of proteins has mainly focused on increasing the stability of enzymes. Shaw, Biochem. J. 246, 1-17 (1987); Ackers and Smith, Ann. Rev. Biochem. 54, 597-629 (1985); Leatherbarrow and Fersht, Protein Engineering 1,
- 20 7-16 (1986). This technique has been used to probe the relationship of structure and function in various proteins. Creighton, Meth. Enz. 131,83 (1986); Falke and Koshland, Science 237, 1591 (1987); Matsumura and Matthews, Science 243, 792 (1989). Random
- 25 immunoglobulin dimers, derived from chemically crosslinked non-immune rabbit immunoglobulin in the absence of antigen has been reported to display some constitutive activity. In complement fixation, the higher order aggregates are significantly more effective
- 30 while monomers were inactive. Wright et al., Biochem. J. 187, 775 (1980). Earlier experiments imply that a higher order complex is required. Hyslop et al., J. Exp. Med. 131, 783 (1970); Baradun et al., Vox. Sang. 7,
- 35 157 (1962). A synergistic effect in complement dependent lysis with IgG and IgM has been reported. Bennett et al., Immunology, 34, 405 (1978); Howard and Hughes-Jones, Progress Allergy, 45, 1 (1988). The

effect of antigen density on antibody dependent complement mediated cytolysis has been examined theoretically (Gower and Segal, Mol. Immunol. 18, 823 (1981)) and experimentally (Alters et al., J. Immunol. 5 (1989)).

SUMMARY OF THE INVENTION

Oligomeric immunoglobulins are provided for serving as effectors of complement mediated cytolysis. By 10 replacing an amino acid with a cysteine in the constant region of the heavy chain, dimers can be obtained which provide for greatly enhanced complement effector function.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Novel oligomeric immunoglobulin G compositions are provided, where an amino acid in the second or third, preferably third constant domain is replaced with a cysteine, whereby the oligomer may be produced in vitro 20 from isolated monomer or in vivo by coupling during the process of synthesis or processing of the immunoglobulin. The immunoglobulins which are employed are those which have complement effector function, the human immunoglobulins being isotypes 1, 3 and 4. The 25 compositions find use in providing for enhanced cytolysis of cells having epitopes to which the antibody binds. The cysteine may be used as a site for bonding to other linking molecules such as bifunctional compounds, e.g. bis-iodoacetyl imide bis-1,2-maleimido 30 ethylene, or the like, which a time halo, olefin or other functionality may serve to bind the sulfur to act as a bridge.

The mutagenesis occurs at a codon in the second or third domain of the heavy chain, preferably the third,

see Kabat et al., Sequences of Proteins of Immunological Interest, U.S. Dept. of Public Health NIH (1987).

Usually the mutagenesis will occur at a residue above

400, preferably at about 440 to 450, more preferably at about 444.

The mutation may be achieved by any convenient means, where the gene for the heavy chain may be isolated, particularly as cDNA. By employing in vitro mutagenesis, one can introduce the cysteine codon at the appropriate site. Alternatively, one may use a portion of the sequence encoding the antibody, conveniently the 3'-proximal region, where by use of appropriate primers, the sequence may be mutagenized using the polymerase chain reaction. Alternatively, the region containing the codon to be mutagenized may be restricted and a different sequence inserted having the appropriate codon at the appropriate site. The particular manner in which the cysteine is introduced is not critical to this invention and any convenient technique may be employed.

Once the heavy chain has been modified to introduce the cysteine, it may then be introduced into any appropriate host in conjunction with a light chain for expression of the desired immunoglobulin. The genes may be introduced into prokaryotic or eukaryotic hosts, particularly eukaryotic hosts, more particularly mammalian hosts, where the cells may be grown in culture or by employing transgenic mice may be grown in vivo. The light chain may be either a kappa or lambda chain, either the naturally occurring light chain naturally paired with the particular heavy chain, or a different light chain, when a different binding affinity may be of interest.

A wide variety of vectors are available for transformation of host cells for expression. The genes encoding the heavy and light chains may be on the same vector or may be co-transformed together. Vectors will normally involve an expression control system.

The host where the expression system may be constitutive or inducible. A wide variety of expression regulatory systems, comprising enhancers,

promoters, and termination signals are available, being derived from viruses, e.g. SV40 early genes, naturally occurring genes, such as beta-actin, etc. Any convenient regulatory system, which provides the desired level of expression and ease of isolation may be employed.

The host cells may be any convenient host cell, particularly mammalian cells, such as monkey cells, mouse cells, etc. Usually, a marker will be provided for detection of cells which have been transformed with the genes encoding the heavy and light chains.

The particular manner of transformation is not critical to this invention, any convenient method being satisfactory. Once the cells have been transformed, they then may be selected in accordance with the nature of the marker and surviving cells cloned and expanded by conventional techniques. The cells may then be grown in culture and depending upon whether the signal sequence has been retained for the heavy chain, the immunoglobulins may be harvested from the supernatant or the cells harvested, lysed and the immunoglobulins separated from the cellular debris.

The mutagenized gene may be either the genomic gene or the cDNA gene. In the case of the genomic gene, the gene may be excised, the appropriate exon isolated and mutagenized, followed by reinserting the exon into its original site. For the cDNA, a fragment may be removed and mutagenized as by in vitro mutagenesis or the polymerase chain reaction, whereby the mutagenized gene may be obtained.

The gene will normally comprise a signal sequence for secretion of the immunoglobulin, the variable regions specific for binding to a particular epitope in conjunction with the light chain, the unmutagenized

first constant region domain, the second constant region domain, the third constant region domain, normally mutagenized and comprising the cysteine. While, for the most part,

the cysteine will be a substitution for another amino acid, it may also be an insertion at an appropriate site. Normally, the transmembrane integrator sequence will not be present, so that the immunoglobulins which
5 form will be capable of secretion and will not be translocated to the membrane. The light chain will normally be the wild type light chain.

For the most part, the immunoglobulins will be human immunoglobulins, although the immunoglobulins may
10 be from any mammalian host, particularly murine, feline, canine, equine, ovine, porcine, lagomorpha or primate. The dimeric antibody can be obtained directly from the expression host. However, it is found that the dimeric antibody is frequently obtained in conjunction with the
15 monomeric antibody. By oxidizing the monomeric antibody in an appropriate aqueous medium, oligomers of the antibody may be obtained. The antibody compositions may be purified by conventional techniques, such as size-exclusion chromatography, gel electrophoresis, affinity
20 chromatography, HPLC, etc.

The oligomeric antibody compositions may be used in a variety of ways. Of particular interest is the use of the subject antibody compositions in conjunction with a source of complement for cytolysis, where the antibodies
25 bind to an epitope of a surface membrane molecule, particularly protein, saccharide, or lipid or combination thereof. The subject oligomers may be also used in therapy for binding to pathogens. In the case of microorganism cells, where the microorganisms are not
30 invasive, the subject oligomers may be directed to epitopes on the surface of the organism. Thus, the subject oligomers may be used for the treatment of various pathogenic diseases, resulting from bacteria, fungi, protista, and the like. Where the organism is
35 invasive, the subject oligomers may be used to bind to the surface of the organism but are translocated to the surface membrane of the invaded cell. In this way, infected cells may be

...the subject oligomers may be used to bind to the surface of the organism but are translocated to the surface membrane of the invaded cell. In this way, infected cells may be

killed, so as to avoid the proliferation and expansion of the pathogen. The subject compositions may also be used in the treatment of neoplastic cells, in both solid and non-solid tumors.

- 5 The subject compositions may be administered in conventional ways normally employed with antibodies. The subject compositions may be formulated in physiologically acceptable media, e.g. water, saline, phosphate, buffered saline, aqueous alkanol, or the
10 like. The amount of the subject oligomers which is employed may vary widely, depending upon the particular purpose, the manner of administration, the frequency of administration, and the like. Generally, from about 1 pg to 10 mg per kilogram of host will be employed. The
15 administration will normally be parenteral, particularly intravascularly, intramuscularly, or subcutaneously. The subject compositions may be administered as the only active ingredient or in combination with other physiologically active compounds.
- 20 The following examples are offered by way of illustration and not the way of limitation.

EXPERIMENTAL

Mutagenesis and an expression to yield the C444 mutant.

- 25 A genomic clone containing the constant domain exons of the heavy chain of human IgG₁ was pared down to a 2.3kb SallI - SphI fragment. This was inserted into pUC18 vector, in which the KpnI and SacI sites had been deleted in the polylinker. A SphI-ClaI-BamHI-(SphI)
30 adaptor was inserted into the downstream SphI site. Insertion of a KpnI linker was used to delete a NaeI site between the C_H2 and C_H3 exons. A NaeI - NsiI fragment at the 3' end of the coding region of the C_H3 exon was replaced by a synthesized fragment which

Cys⁴⁴⁴. The resulting vector, pU18G1C444, was sequenced and the desired mutation verified. An undesired one

base deletion occurred. However it was 3' of the stop codon and would not affect protein synthesis. The mutagenized C₃ exon was cloned with the wild-type C₁' hinge and C₂ Hexons into a mammalian expression vector, pSV2DNSG1, which contained the heavy chain variable exon for anti-dansyl specificity. The resulting mutagenized heavy chain expression vector, pSV2DNSG1C444, was co-transfected into the murine myeloma SP2/0 cell-line with a mammalian expression vector coding for the anti-dansyl light chain with a human kappa exon. The resulting clonal cell-line, 143-17.12, produced a mutant dansyl-binding antibody with human constant domains of γ_1 and kappa isotype (C444). Antibody was purified from three liters of supernatant, at approximately 3 mg/L, by affinity chromatography, dialysis and size-fractionation. Parental antibody, an anti-dansyl human γ_1 , kappa with mouse variable domains (huG1), was produced in a similar manner. Cloning, sequencing and transfections were done as described by Maniatis et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Publications, 1982); Biggin et al., Proc. Natl. Acad. Sci. USA 80, 3963 (1983); Morrison and Oi in Handbook of Experimental Immunology (Weir ed), Vol. 2, 92.1 (Blackwell Scientific Publications, Palo Alto 1986).

Analysis of C444 Mutant by SDS-PAGE and gel-filtration chromatography.

A comparison was made of the C444 mutant and parental HuG1 by SDS-PAGE. The parent and mutant protein were run with no reductant on a 6% gel with high molecular weight standards. In a second run the two proteins were reduced with 20 mM dithiothreitol and run on a 10% gel with low molecular weight standards. The resulting gels were stained with Coomassie Blue, washed with 1% acetic acid, and then developed with 0.5% silver nitrate. The gels were then run on a Superose 12 column (Pharmacia). Running buffer was 50 mM Tris,

150 mM NaCl (pH 7.8) at 0.25 ml/min. Native molecular weight standards and monomeric parental HuG1 ($\gamma\epsilon$) were run under the same conditions.

5 Antibody dependent complement mediated cytotoxicity
 measurements.

 Titration of either parental HuG1 or isolated C444 dimer on sheep erythrocytes which were previously surface-labeled with dansyl₂₈-BSA by a CrCl_3 method was performed (Mishell and Shiigi, Selected Methods in Cellular Immunology (W.H. Freeman and Co., San Francisco 1980). Antibody (10 μl) and sensitized cells (10 μl at 4×10^6 cells/ml) were incubated at 23°C for 30 minutes in a U-bottom 96-well plate. Guinea pig complement (10 μl of an 1:1 dilution of Hemo-Lo (Cedarlane)) was added and incubated at 37°C for one hour. The cells were washed twice with buffer, lysed with 200 μl water and split in four equal volumes into a flat-bottom 96-well plate. A chromogenic substrate for esterases, 0.1 mg/ml fluorescein diacetate (Molecular Probes), was added in a 1:1 volume. After development at 37°C for 1-2 h, the O.D. at 490 nm was read by a V-Max microplate reader (Molecular Devices). The O.D. reading was converted to number of cells and the percent lysis calculated. (Need more detail on the procedure). The buffer in all dilutions was 1x gel-HBS (Hardy, supra). Immunoglobulin was size-purified on a Superose column just prior to the experiment.

RESULTS

30 The C444 HuG1 antibodies were comprised of a 26kD (kilodalton) light and 53kD heavy chain as shown by SDS polyacrylamide gel electrophoresis (SDS-PAGE) of fully reduced samples. 50% of secreted C444 was typical immunoglobulin monomer, identical in size to the remainder. The remainder was consistent with a covalent dimeric immunoglobulin. Non-denaturing, non-reducing gel-filtration chromatography

of C444 also indicated two distinct forms consistent with monomer and dimer forms of immunoglobulin. Upon reduction with 10 mM dithiothreitol, the C444 dimer was converted to monomeric form. Removal of the reductant by size exclusion chromatography allowed the formation of the C444 dimer. The data support that the dimeric form of C444 is attributable to a reducible bond. Pepsin digestion of the dimeric C444 indicated the formation of novel covalent but reducible, bonds between carboxy terminal domains. Cleavage of human IgG1 at residue 333 with pepsin yields a pFc' fragment which corresponds to essentially the carboxy terminal domain (Stanworth and Turner, in Handbook of Experimental Immunology (Weir ed.), Vol. 1, 12.1 (Blackwell Scientific Publications, Palo Alto 1986)). Digestion of C444 dimer with pepsin gave only a fragment corresponding to (pFc')₂, while the parental IgG1 yielded a fragment corresponding to pFc'. However, the fragments became identical upon reduction. Thus the bond in the dimer was indeed between C_H3 domains. The absence of any pFc' derived from the dimer indicated that the majority of C444 dimers are linked via two, rather than one, bond.

The data would support that the introduced cysteines at residue-444 in a pair of immunoglobulins forms an intermolecular disulphide linkage leading to a "dog-bone" shaped dimer.

The higher molecular weight form of the C444 mutant could be preparatively isolated by non-denaturing gel-filtration. This was shown to be equal to greater than 90% dimer by SDS-PAGE analysis, did not appear to contain oligomers greater than dimer and was stable for many weeks at 4°C. Isolated C444 dimer was used in an antibody dependent complement mediated cytotoxicity assay

indicative that the monomer was not active against target cells. Quantitatively, an initial concentration of 0.3 µg/ml of the C444 dimer gave 66% cytotoxicity. To

attain the same level of cytolysis with the parental HuG1, an initial concentration of 30 $\mu\text{g/ml}$ was required; a 200-fold increase in dosage on a molar basis.

5 The effectiveness of the dimer was shown not to be simply a mass action effect due to its tetravalent nature. At initial concentrations between 5 to 0.5 $\mu\text{g/ml}$, where the complement activity difference is the greatest, the amount of C444 dimer bound to cell-
10 surface antigen was less than 10% greater than that of HuG1 monomer and this negligible difference cannot account for the degree of lysis observed. Nor can the effect be due to the replacement of the Ser⁴⁴⁴ residue in the mutant as monomeric C444 gave the same lysis
15 results as monomeric HuG1. The data supports the conclusion that the greater activity of the dimer is attributable to the greater efficiency in the initiation of the complement cascade that leads to cell lysis.

For the purpose of comparison with immunoglobulin
20 aggregation, chemically cross-linked monoclonal HuG1 anti-dansyl dimers derived from the parental monomers were prepared. Randomly linked anti-dansyl immunoglobulin dimer was prepared from monomer parental HuG1 by chemically cross-linking with the
25 homobifunctional reagent SMPC (Pierce) (method B in Wright et al. (1980), supra) and isolating a 300 kD fraction by size-exclusion chromatography. In the presence of complement, the random cross-linked dimer at initial concentrations from 1 to 10 $\mu\text{g/ml}$ induced lysis
30 of less than 10% ($\pm 5\%$) of the labelled cells. This is significantly less than the 100% ($\pm 5\%$) lysis given by the C444 dimer or even the 48% ($\pm 8\%$) lysis observed for monomeric HuG1 at the same dosage.

The ability of the dimer to consume complement in

this assay monomeric immunoglobulin requires the binding of antigen for the formation of immune complexes to

initiate complement fixation. A small amount of pre-aggregated immunoglobulin, less than 5% of the total, can fix complement in the absence of antigen. In contrast to aggregated immunoglobulin, the amount of complement consumed constitutively by the C444 dimer was negligible, less than 5%; even at a 200-fold higher concentration than adequate for complete target cell lysis. The C444 dimer was effective at complement consumption in the presence of antigen.

It is evident from the above results, that greatly enhanced levels of in cytolysis of cells may be achieved by employing the oligomers of the subject invention. By providing for specific linking at a site in the constant region, particularly the C_H3 domain, substantially smaller amounts of the subject compositions may be employed while achieving complete cytolysis. Thus, more efficient removal or destruction of cells can be achieved with the subject compositions as compared to monomeric immunoglobulin.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. An oligomeric immunoglobulin comprising at least two immunoglobulin molecules other than IgM comprising a cysteine in the heavy chain constant region as a result of a mutation at a codon greater than the 350th codon, wherein said immunoglobulin molecules are joined by mean of said cysteine to form an intermolecular link.
2. An oligomeric immunoglobulin according to Claim 1, wherein said immunoglobulin molecules are IgG.
3. An oligomeric immunoglobulin according to Claim 2, wherein said IgG is of an isotype having complement effector function.
4. An oligomeric immunoglobulin according to Claim 2, wherein said mutation is substitution of a amino acid at a residue in the range 440 to 450.
5. An oligomeric immunoglobulin according to Claim 4, wherein said residue is 444.
6. A DNA sequence encoding an immunoglobulin heavy chain subunit of other than IgM comprising a cysteine as a result of a mutation at a codon greater than the 350th codon.
7. A DNA sequence according to Claim 6, wherein said immunoglobulin molecules are IgG.
8. A DNA sequence according to Claim 7, wherein said IgG is of an isotype having complement effector function.
9. A DNA sequence according to Claim 7, wherein said mutation is substitution of a codon at a residue in the range 440 to 450.
10. A DNA sequence according to Claim 9, wherein said residue is 444.
11. A DNA sequence according to Claim 6 comprising at the 5' terminus a signal sequence.

13. An expression cassette comprising in the order of transcription, a promoter, a DNA sequence encoding an immunoglobulin heavy chain subunit of other than IgM comprising a cysteine as a result of a mutation at a codon greater than the 350th codon, and a terminator sequence.

14. An expression cassette, wherein said DNA sequence encodes a signal sequence at its 5' terminus.

15. A eukaryotic host comprising an expression cassette according to Claim 14.

16. A eukaryotic host comprising an expression cassette according to Claim 13.

17. A method of preparing an oligomeric immunoglobulin comprising at least two immunoglobulin molecules other than IgM comprising a cysteine in the heavy chain constant region as a result of a mutation at a codon greater than the 350th codon, wherein said immunoglobulin molecules are joined by said cysteine to form an intermolecular cystine, said method comprising:
growing a eukaryotic host according to Claim 16 in an appropriate nutrient medium, whereby said oligomeric immunoglobulin is expressed; and
harvesting said oligomeric immunoglobulin.

18. A method for producing complement mediated cytolysis, said method comprising:
bringing together (1) cells to be cytolysed, (2) an oligomeric immunoglobulin comprising at least two immunoglobulin molecules other than IgM comprising a cysteine in the heavy chain constant region as a result of a mutation at a codon greater than the 350th codon, wherein said immunoglobulin molecules are joined by said cysteine to form an intermolecular cystine, said oligomeric immunoglobulin being of an isotype to bind complement and specifically binding to a surface

complement bind to said cells and lyse said cells.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/04395

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): A61K 39/395, C07K 13/00; C12N 15/13; U.S.C1: 530/387; 536/27; 435/69.6		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
U.S.C1:	435/69.6, 965, 971, 972, 975; 530/387-390, 408; 536/27; 424/85.8, 85.91, 529-534	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
Automated Patent System and Dialog Keyword Searches: complement?, mutant?, chimeric?, igl, immunoglob?		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	FASEB, vol. 4, issued 07 June 1990, Baird et al., "IgE has a bent conformation before and after binding to its high affinity receptor (F _c E RI) on RBL cells", see abstract 2153.	1-18
Y	DE, A, 3,112,334 (Becker et al) 07 October 1982, see entire document.	1-18
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search:		Mailing of this International Search Report:
<u>23 September 1991</u>		<u>08 OCT 1991</u>
International Searching Authority		Signature of Authorized Officer
<u>ISA/US</u>		<u>Terence Marie</u> T. Michael Nisbet